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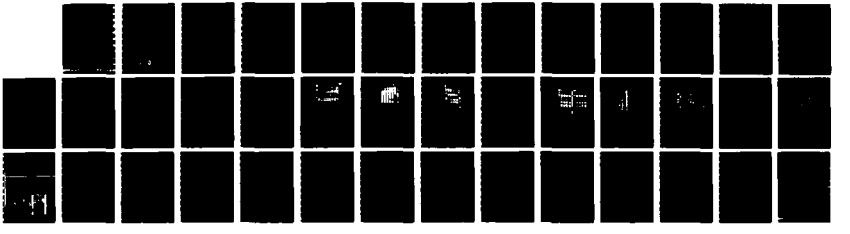

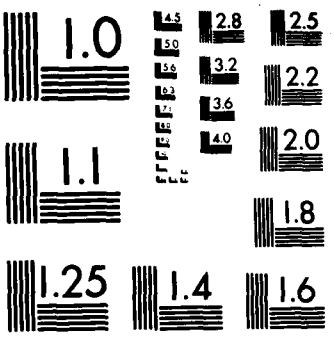

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PROGRESS REPORT

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AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
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"Cloning of the poly(ADP-ribose) gene" from Rat Liver

Submitted September 24, 1986

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On-going research (supported by AFOSR F08671-8600478) demonstrated that inhibition of the nuclear enzyme poly (ADP-ribose) polymerase by substances that interfere with the DNA binding of the enzyme molecule profoundly inhibit cellular phenotypic changes (malignant transformation) induced either by non-toxic doses of ultimate carcinogens and more recently in an oncogene construct-containing cell line by steroid hormones. Enzyme inhibition in ontogenically stable cells had no measurable physiological effect as tested by cell growth or viability, thus the biological role of poly (ADP-ribose) polymerase seems to be confined to cells undergoing differentiation, development or oncogenesis that occurs without detectable cellular toxicity (no DNA damage).

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4. DNA synthesis by DNA-synthesizer: Cary Levinson, Ph.D. (Cetus Corp., Berkeley).
5. Amino acid analysis done in UCSF Bioanal. Lab.

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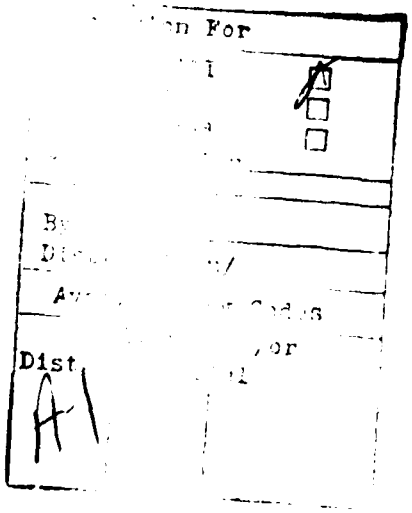
Purpose of Research and Summary of Progress Made in One Year

On-going research (supported by AFOSR FQ8671-8600478) demonstrated that inhibition of the nuclear enzyme poly(ADP-ribose) polymerase by substances that interfere with the DNA binding of the enzyme molecule profoundly inhibit cellular phenotypic changes (malignant transformation) induced either by non-toxic doses of ultimate carcinogens and more recently in an oncogene construct-containing cell line by steroid hormones. Enzyme inhibition in ontogenically stable cells had no measurable physiological effect as tested by cell growth or viability, thus the biological role of poly(ADP-ribose) polymerase seems to be confined to cells undergoing differentiation, development or oncogenesis that occurs without detectable cellular toxicity (no DNA damage).

This unique feature of the enzyme made it necessary to focus attention on the molecular and genetic properties of poly(ADP-ribose) polymerase, an effort that complements on-going research on carcinogenesis and its prevention (AFOSR FQ8671-8600478). MOLECULAR CLONING DEPENDS ON THE ISOLATION OF THE GENE PRODUCT (ENZYME) AND EVENTUAL ISOLATION OF THE DNA THAT CODES FOR THE PRODUCT POLY(ADP-RIBOSE) POLYMERASE FOLLOWED BY PRODUCTION OF DNA PROBES WHICH CAN IDENTIFY AND QUANTITATE THE GENE IN INTACT CELLS UNDER VARYING PHYSIOLOGICAL AND TOXICOLOGICAL CONDITIONS. THE ENSUING TECHNIQUE WILL HAVE THE POWER OF DETECTION OF THE GENE IN INDIVIDUAL CELLS.



The following report contains two methods of isolation of homogeneous poly(ADP-ribose) polymerase protein. The first one is

a modification of earlier procedures and the second method representing a new approach, utilizing affinity precipitation, that within 3 working days produces pure enzyme with about 20% recovery. This method superceeds any other procedure by a factor of at least 5 to 10. Antibodies against the enzyme were generated in rabbits and an immunotransblot technique developed for enzyme quantitation in biological materials. The amino-acid sequence of the enzyme has so far been determined because the amino terminal is blocked. We for the first time obtained a peptide fragment of 36 kDa size that was sequencable. This peptide binds DNA, this represents a critical part of the enzyme. Several shorted peptides were also isolated and sequenced.

A sequence of 51 AA was obtained by plasmin digestion and sequencing and DNA probes for two 12 AA sequences were synthesized.

These DNA probes are now applied for the search of the polymerase gene in genomic libraries (second stage of investigation). Availability of DNA probe are a critical requirement for the ultimate isolation of the gene and will facilitate the second experimental approach following the mRNA-cDNA scheme (as described in the original grant proposal).

METHOD I

INTRODUCTION

Poly(ADP-ribose) polymerase enzyme is an eukaryotic nulcear protein that can be extracted with 0.35 M salt. Papers describing

purification procedures invariably use this first step. The subsequent steps of purification, however, vary considerably from paper to paper. Some use ammonium sulfate fractionation of the crude extract (Yoshihara, 1978) some do not (Ito, 1979). Another step of purification depends on affinity chromatography. Earlier procedures use exclusively DNA-cellulose (Yoshihara, 1978) or DNA-agarose (Ito, 1979) that depends on the DNA-binding property of this enzyme. In later papers blue-sapharose (Holtlund, 1983) or red sepharose (Zahradka, 1984) are applied in succession with DNA-columns. These methods depend on matrix bound dyes which are NAD analogs and serve as ligands for the enzyme. In a recent paper, benzamide-agarose was described as an affinity adsorbent for the enzyme, but with admittedly low yield (Burtscher, 1986). In this latter paper, the early application of hydroxylapatite adsorption renders it hard if not impossible to recover coenzymic DNA.

It is essential that a reproducible isolation method be developed. We found the highly substituted reactive red$^{\underline{120}}$ - agarose very reliable in an early step of purifying poly(ADP-ribose) polymerase enzyme, but it required ethylene glycol in the buffer to prevent nonspecific hydrophobic interactions and consequent denaturation of the enzyme protein. This step circumvented the often unreliable DNA-cellulose adsorption step.

We also describe another improvement here. Instead of the time consuming gel-filtration or the poorly resolving phosphocellulose chromatography, we introduce fast protein liquid chromatography (FPLC) on MONO-S (Pharmacia) resin as the final step of purification.

Poly(ADP-ribose) polymerase enzyme has a relative molecular mass of 115 kd (Burtscher, 1986). Since it runs as a single polypeptide on SDS-PAGE , it must contain about 1000 amino acid residues. Amino acid sequencing on it would be a major undertaking. The logical choice is cloning its gene and sequencing its DNA. To that end, one requires DNA probes for screening DNA libraries, and for the synthesis of DNA probes one needs amino acid sequences from the protein itself. The amino acid composition of the enzyme has been determined in various laboratories (Pekala, R. Moss, 1983) but no sequences were reported because the amino terminal is blocked. Proteolytic splitting and isolation of two halves, assigning functional domains to them has been published (Kameshita, 1986). In that paper, papain and chymotrypsin were utilized to split the protein but these workers did not report sequences either. To get amino acid sequences of known function, isolation of peptides is a prerequisite. These peptides should be of defined size, suitable for sequencing but also large enough for the identification of specific functions (e.g., DNA binding). We found plasmin as a suitable enzyme for this purpose. It hydrolyzed poly(ADP-ribose) polymerase into large peptide moieties, enabling us to obtain sequences for the DNA-binding region. The present report is concerned with this problem.

MATERIALS

Frozen calf thymus was obtained from Roth Products, Inc., Route 63, & US Route 202, DeKalb Pike, Gwynedd, Pennsylvania 19436.

Reactive red 120-agarose #R0503, Sigma (Lot 84^{I}-9625) contained 5.1 uM red dye per ml packed gel.

Hydroxyapatitie: Biogel HTP from Bio-Rad.

Plasmin #P7911, Sigma 3.6 units/mg; reconstituted in distilled water 1.4 mg/ml; kept frozen in small aliquots until use.

[^{32}P]-NAD^{+} from NEN Research Products, Boston, Ma.

All other reagents were of the highest quality available.

Anti-poly(ADP-ribose) polymerase antiserum was raised in rabbits injecting the enzyme with Freund's complete adjuvant, then administering monthly boosters. The titer of antiserum used was above 10^5.

Anti-rabbit IgG #A8275 Sigma.

FPLC was done on Mono S HR 5/5 cation exchanger columns (Pharmacia) with Beckman 421 controller, Beckman-Altex 100 A pumps, and Beckman-Altex 155 variable wavelength detector.

Peptide hydrolysates were analyzed by the Picotag method using the Waters 440 system.

Sequencing of the amino terminals of peptides was performed on an Applied Biosystems, Inc. model 470A gas phase sequencer with on line 120A PTH analyzer (Hunkapillar, 1983) at UCSF, BRC by Andras Patthy.

Buffer and Solutions:

PBS: 10 mM phosphate, 150 mM NaCl, pH 7.2.

TBS: 25 mM Tris-HCl 150 nM NaCl, pH 7.3.

Blocking buffer for immunoblots: PBS containing 1% bovine serum albumin (BSA) and 0.1% of NaN_3.

Washing buffer for immunoblots: PBS containing 0.05% Tween 20 and 0.01% Thimerosal.

Buffer for digestions: 25 mM HEPES, 150 mM NaCl, and 10 mM mercaptoethanol, pH 8.5.

Phenylmethylsulfonyl fluoride (PMSF) stock solution was made in abs. EtOH at 100 mM, and added to buffers immediately before use to obtain a final concentration of 0.1 mM.

Extraction buffer: 50 mM Tris-HCl, 10 mM EDTA, 1 mM NaN_3, 50 mM $NaHSO_3$ 10 mM mercaptoethanol and 0.3 M NaCl, pH 7.4.

Buffer A, 50 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, 1 or 2 M KCl, 10 mM mercaptoethanol, 1.0, 50, or 300 mM phosphate, and 10% glycerol.

Buffer B, 50 mM Tris-HCl, 50 mM NaHSO3, 1 mM EDTA, 1 mM NaN_3, 10 mM mercaptoethanol, 10% glycerol, 15% ethylene glycol, 0.02 or 2.0 M NaCl as indicated.

Assay of poly(ADP-ribose) polymerase:

The assay mixture contained 25 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM [^{32}P]-NAD^+ (10-30000 cpm per µl) and 2 µg coenzymic DNA in final volume of 50 µl. The last component was omitted from assays of enzymes having coenzymic DNA still attached, i.e., before the hydroxyapatite elution step.

Assay mixtures were incubated for 3 min. at 25°C and the reaction was stopped with 1 ml of 10% TCA. Precipitates filtered onto Whatman GF/C glass filter discs, washed with 5 x 2 ml 10% TCA, then with ethanol. Their radioactivity counted in plastic scintillation vials using Betamax (West Chem., San Diego, Ca.) scintillation mixture in a Packard beta counter. The enzyme unit was defined as nmole ADP-ribose incorporated per minute under conditions defined above.

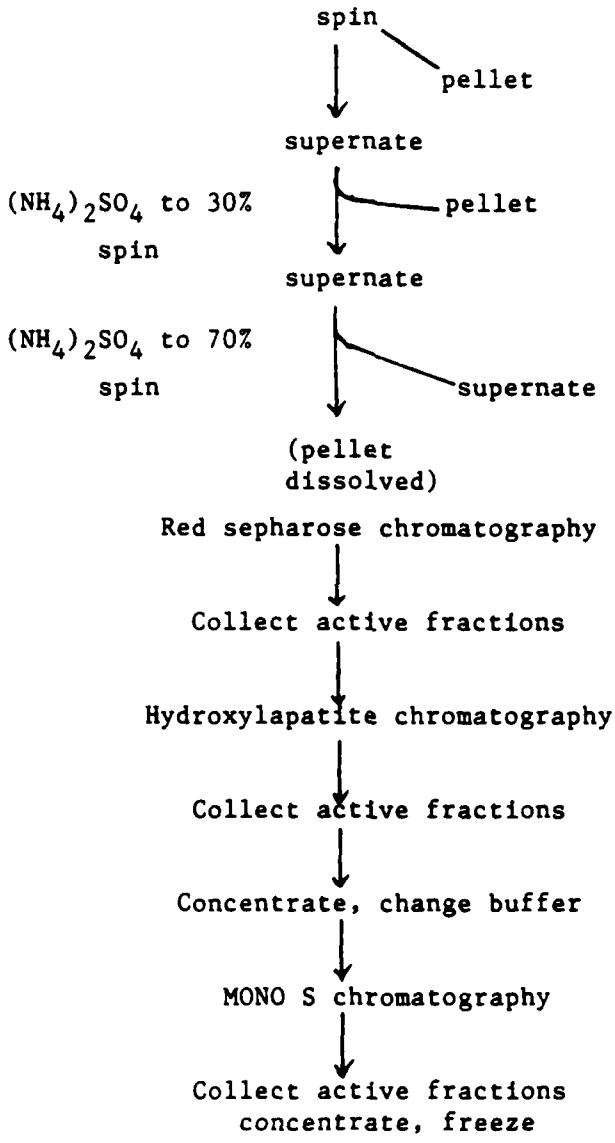
Immunoblotting was performed according to Burnette (1981) and SDS-PAGE was done following Laemmli (1970) in 10% polyacrylamide gels. After electrophoresis, gels were immediately transferred into a Bio-Rad transplot tank, peptides transblotted to nitrocellulose membranes (anode side) in an electrode buffer consisting of 192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3; applying 100 volts for one hour. The gel slab was stained with coomassie blue for remaining protein bands. The surface of nitrocellulose membrane was blocked with blocking buffer for 1 hour at room temperature then washed with washing buffer. Incubation with antiserum against poly(ADP-ribose) polymerase enzyme was performed with 1000 fold diluted antiserum in the washing buffer for one hour at 37°C. This was followed by 5 washings and incubation with 1000 x diluted peroxydase-labelled anti-rabbit goat serum (Sigma) for one hour at 37°C. After 5 washings, the nitrocellulose membrane was covered with 20 ml of 1 mg/ml 3,3′-diaminobenzidine, 20 µl concentrated H_2O_2 added, incubated for 3-5 minutes, then washed with distilled water. Immunoreactive peptides were visualized as brown hands.

Protein concentrations were determined by the Bradford (1979) dye binding assay.

Pyroglutamate aminopeptidase digestion. 50 µg C peptide (see Fig. 8) was incubated with 10 units of pyroglutomate aminopeptidase (Sigma #P9516) in 0.1 M bicarbonate buffer pH 7.7 for 60 minutes at 37°C. After incubation the mixture was injected into MONO S column and the C peptide reisolated, for Edman degradation.

Flow Sheet of Purification

Frozen tissue → crushed → homogenized in extraction buffer strained through cheese cloth



RESULTS

Isolation of poly(ADP-ribose) polymerase.

The whole procedure was carried out at 0-4°C except the FPLC chromatograpy, which was run at room temperature.

Extraction: 2 x 250 g frozen (-70°C) calf thymus (with connective tissue and fat) was broken into pea-sized pieces in a high-walled wooden box. The broken pieces were transferred into a blender containing 3 volumes of extraction buffer, homogenized for 1 min. and filtered through 2 layers of cheesecloth. The residue was re-extracted with 1 volume of extraction buffer and filtered. The combined extracts were centrifuged at 7000 x g for 15 minutes. The thick lipid layer on top was removed by aspiration and the supernatant saved for fractionation with ammonium sulfate.

Precipitation with ammonium sulfate.

To the supernatant containing the enzyme, solid ammonium sulfate was added to attain 30% saturation, followed by stirring for 30 minutes and the precipitate sedimented at 7000 x g for 15 minutes. The supernatant, containing the enzyme, was brought to 70% saturation with solid ammonium sulfate and stirred for an hour and again centrifuged at 7000 x g for 30 minutes. The pellet containing the enzyme was dissolved in buffer B containing no NaCl.

Reactive red 120-agarose chromatography.

The reactive red-agarose column (5 x 20 cm) was equilibrated with buffer B containing 0.2 M NaCl. The 30-70% ammonium sulfate cut was diluted to a conductivity equal to that of the equilibrating buffer and applied to the red-column. The column was then washed with the equilibrating buffer and eluted with a salt gradient between 0.2-2.0 M

in buffer B (2 litres). Fractions containing poly(ADP-ribose) polymerase activity were pooled for the next step.

Hydroxylapatite chromatograpy.

To the pooled fractions obtained as described above, 10 g (dry weight) hydroxylapatite was added as a thick slurry made up in buffer A containing 1 M KCl. It should adsorb the enzyme immediately (if not, more hydroxylapatite was added). The settled hydroxylapatite was poured into a wide short column, washed with buffer A containing 1 mM phosphate and 2 M KCl, and eluted with a phosphate gradient between 1 to 50 mM in the same buffer.

Coenzymic DNA adsorbes on the hydroxylapatite and can be eluted with a phosphate gradient between 50 to 300 mM (Yoshihara, 1978). The fractions containing polymerase activity were pooled, concentrated in an Amicon concentrator cell with PM30 membrane. The medium was exchanged at the same time to 2.5 mM HEPES, 100 mM NaCl, 10 mM mercaptoethanol, pH 7.7, which is the starting buffer of FPLC.

Fast protein liquid chromatography. (FPLC) on MONO S column.

Aliquots (5 to 8 µg protein) of the concentrated enzyme from the hydroxylapatite eluate was injected into MONO S and eluted with a gradient of 100 to 1000 mM NaCl in HEPES buffer (pH 7.7). The flow rate was 0.8 ml per minute and the gradient length was 60 minutes. The active peak emerged around 0.3 to 0.35 M NaCl. It was collected by manual fraction changing, as monitored by absorbancy changes at 280 nM. The enzyme was concentrated to 3.5 mg protein per ml, glycerol were added to a final concentration of 10% and 20 mM respectively. The concentrated enzyme solution was then distributed in convenient aliquots

TABLE I

Typical Purification of poly(ADP-Ribose) Polymerase from (500 g of calf thymus wet weight)

	Vol(ml)	U/ml	Protein mg/ml	mg	Total	Total Protein (mg)
ammonium sulfate precipitate	240	136	19.3	12.4	33200	4632
Red-Sepharose eluate	240	12.5	1	12.5	3000	240
Hydroxylapatite eluate concentrated	12	416	3	139	5000	36
MONO S eluate, final product	0.35	4600	4.7	975	1610	1.64

U = units.

and stored at -70°C. The enzyme showed a single band on SDS-PAGE and had a specific activity of 800-1000 units per mg protein. (Table I).

Proteolytic degradation, isolation of peptides and amino acid sequences.

(a) Time course of digestion and the comparison of trypsin chymotrypsin and plasmin.

Limited proteolysis with plasmin proved to be superior to that with trypsin and chymotrypsin because plasmin leaves large peptides intact. These peptides were large enough for isolation and for assays of catalytic properties, as well as partially sequencing (compare Fig. 1 to 3).

Figure 1 and 2 shows that a multitude of peptides are present right from early time points even at low peptidase-substrate ratios ($\frac{1}{250}$ and 1/1375 respectively). On the other hand, plasmin, being more selective with its substrates splits the enzyme first into larger peptides (Fig. 3). These larger peptides break down only later following prolonged incubation (not shown). The residual poly(ADP-ribose) polymerase activity correlates with the remaining intact enzyme (Fig. 3, 4).

It is of interest that coenzymic DNA does not protect the enzyme from proteolytic attack, except for a small delay in hydrolytic rates (compare 5 minute lanes on Fig. 5).

Chromatography of peptides on DNA-cellulose.

As seen from Fig. 6, there are two peptide bands retained on DNA-cellulose (29 and 36 kd); the bigger fragments (42 kd and 57 kd in size) run through this column unbound with 0.2 M NaCl as eluent. The retained peptides are the most basic ones as identified by their amino acid composition (Table II) and their higher affinity to the

cation exchanger MONO S (Fig. 8, peaks A,B and C; -- peak A and B both are 36 kd peptides).

Chromatography on Reactive Red 120-Sepharose.

The elution profile of plasmin peptides from red-sepharose is shown in Fig. 7. There is only one peptide that does not bind to this adsorbent, the 42 kd (which is actually a doublet on SDS-PAGE). Peptides 36 kd and 56 kd bind least strongly. Peptides 29 kd, 32 kd and a minor component around 57 kd size bind more avidly.

Separation of peptides

The basic peptides from plasmin digest separate well on MONO S (see Fig. 8 and 9). With a linear salt gradient from 50 to 1000 mM NaCl in 25 mM HEPES and 10 mM mercaptoethanol, pH 7.7, we were able to separate three peptides: peaks A, B and C. A and B have the same molecular mass: 36 kd and C is smaller, 29 kd. The rest of the mixture is not well resolved. The 56 kd and 42 kd peptides do not exhibit sharp peaks at 280 mM in the MONO S column. The 42 kd peptide was isolated from the red-sepharose by low salt wash, the 56 kd was purified by rechromatography of its broad peak on the MONO S column.

Antigenicity of peptides obtained by plasmin digestion.

In order to study the antigenic properties of the peptides, they were first separated on SDS-PAGE, then transblotted onto nitrocellulose membrane, visualized with a specific antibody by the peroxydase labelled second antibody technique.

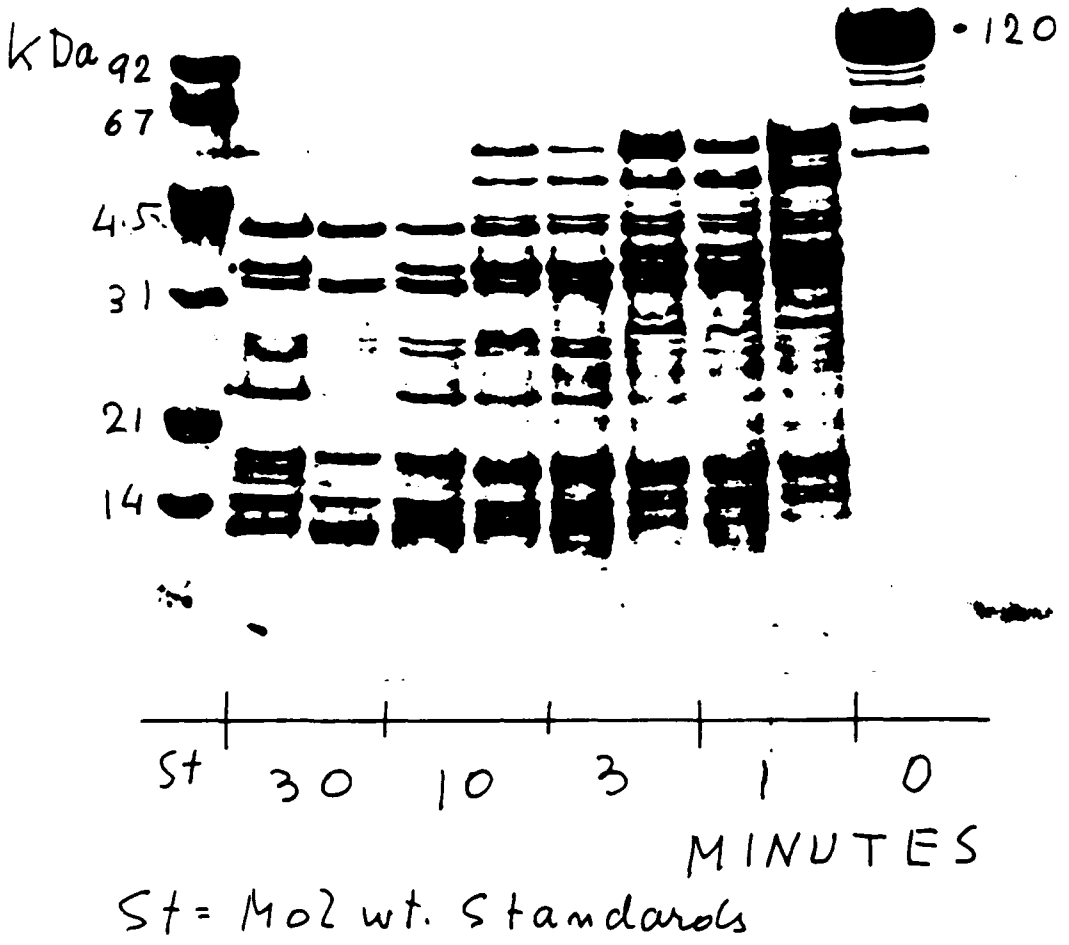
As can be seen on Fig. 10, all of the major large peptides exhibit antigenicity.

TABLE II

% Aminoacid Composition of Plasmin Peptides

Peptides	A 36 kd	B 36 kd	42 kd	C 29 kd
asp	8.813	9.518	6.845	6.569
glu	12.170	12.888	10.184	9.115
ser	7.606	7.976	10.851	9.207
gly	5.116	5.521	14.691	7.822
his	0.569	0.729	1.669	1.772
arg	2.490	2.363	3.840	4.593
thr	5.180	5.244	3.339	4.176
ala	8.575	8.272	11.519	5.449
pro	6.399	6.056	5.008	4.430
try	0.110	0.083	5.008	4.899
val	7.334	6.933	5.008	6.396
met	2.813	2.650	1.669	5.072
cys	0.348	0.526	3.172	1.609
lle	4.683	4.228	2.504	5.408
leu	8.617	8.272	6.678	4.766
phl	2.881	2.742	3.005	5.377
lys	16.368	15.999	5.008	13.341

Figure 1: Time course of digestion of the polymerase with trypsin.

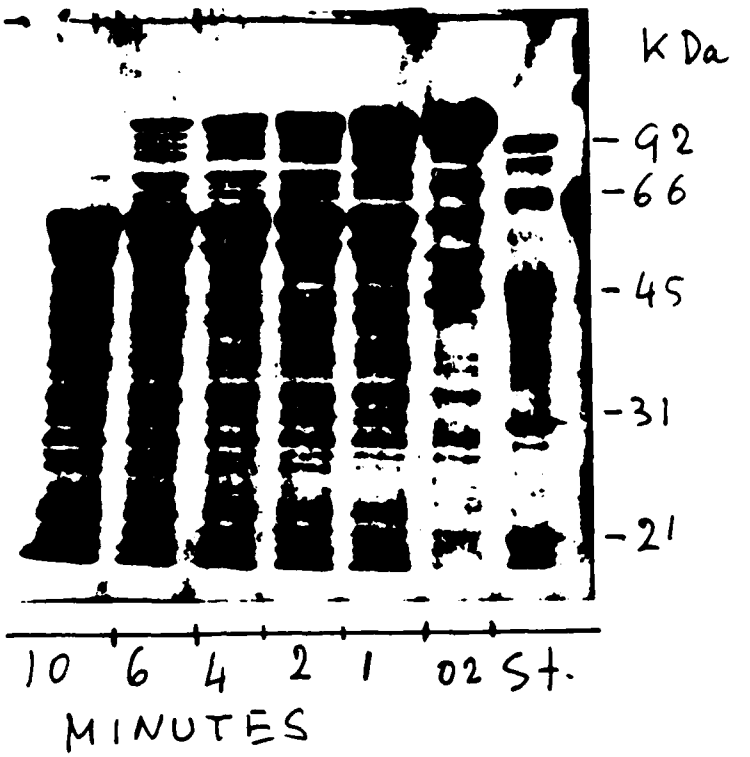


Experimental: $\frac{\text{Enzyme}}{\text{protease}} = 250$

Digestion buffer: 25 mM HEPES, 150 mM NaCl 10 mM SH-EtOH, pH 8.5
3 µl aliquots on SDS-PAGE 12.5% Coomassie stain.

Fig 2.

FIGUR[illegible]: Time course of digestion of [illegible]e enzyme with chymotrypsin.

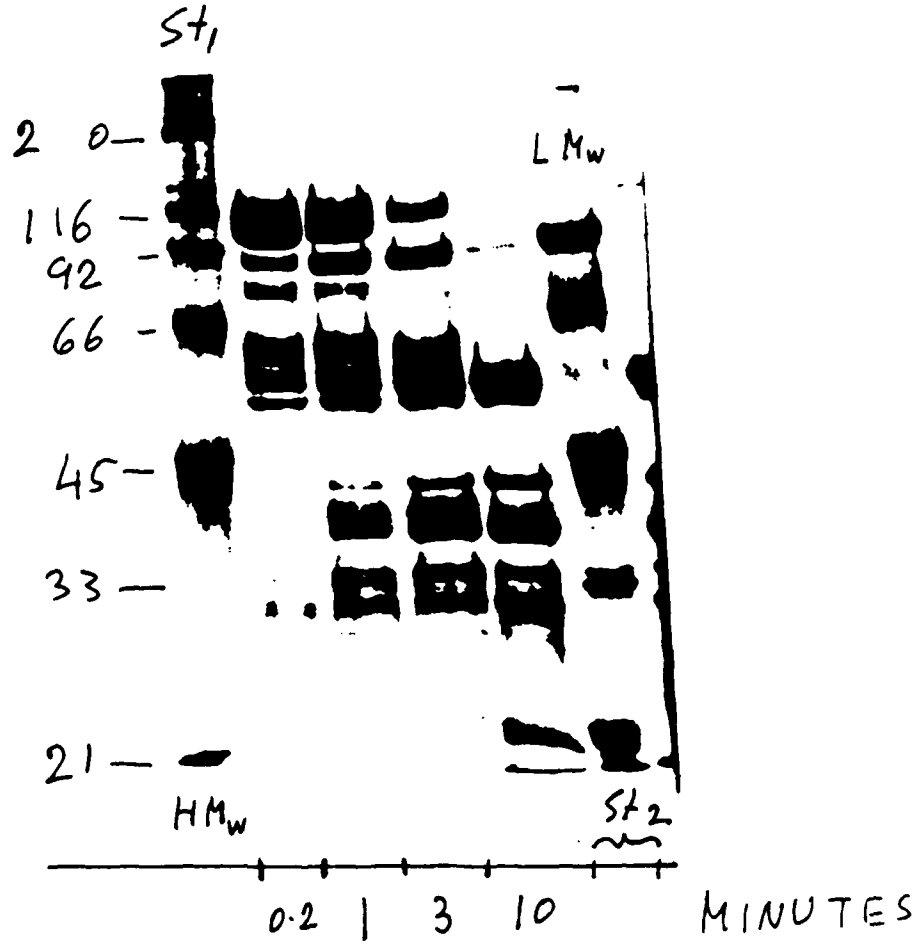


Experimental: $\frac{E}{\text{protease}} = 1375$

pH = 8.0; Digestion buffer: 50 mM Tris, 150 mM NaCl, 10 mM SH-EtOH.

FIGURE 3: Digestion of the enzyme with plasmin.

Fig 3



Experimental: $\frac{E}{protease} = 90$

Conditions same as for Fig. 1, pH 8.5.

Fig 4.

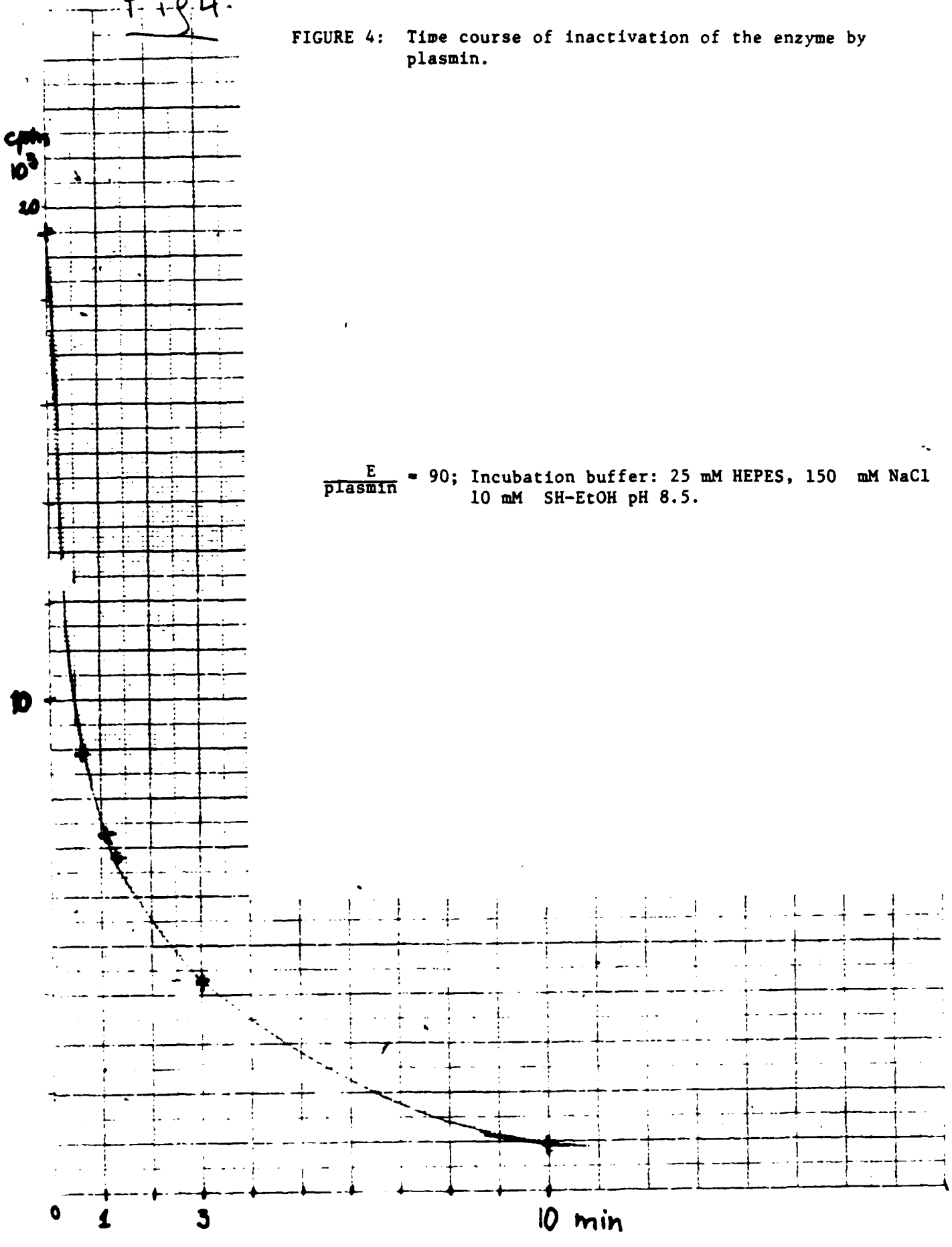
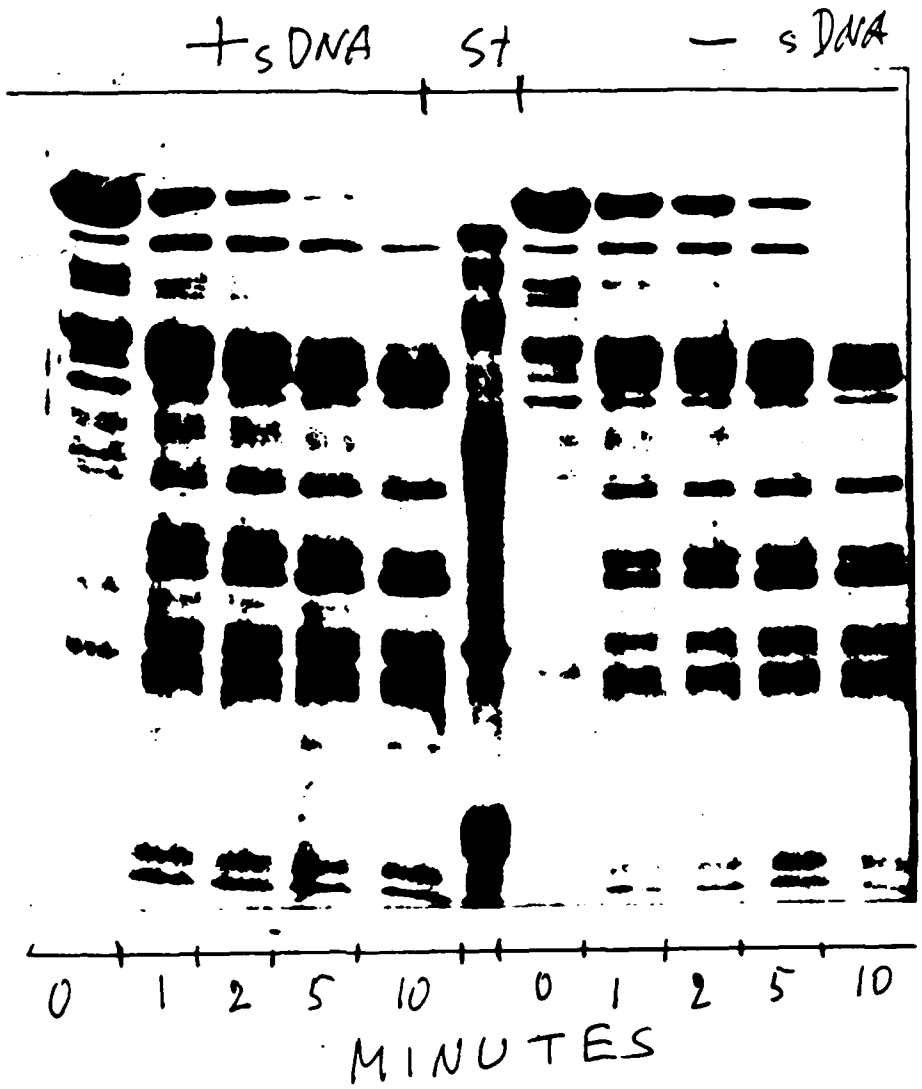


FIGURE 4: Time course of inactivation of the enzyme by plasmin.

$\frac{E}{plasmin}$ = 90; Incubation buffer: 25 mM HEPES, 150 mM NaCl 10 mM SH-EtOH pH 8.5.

Fig 5

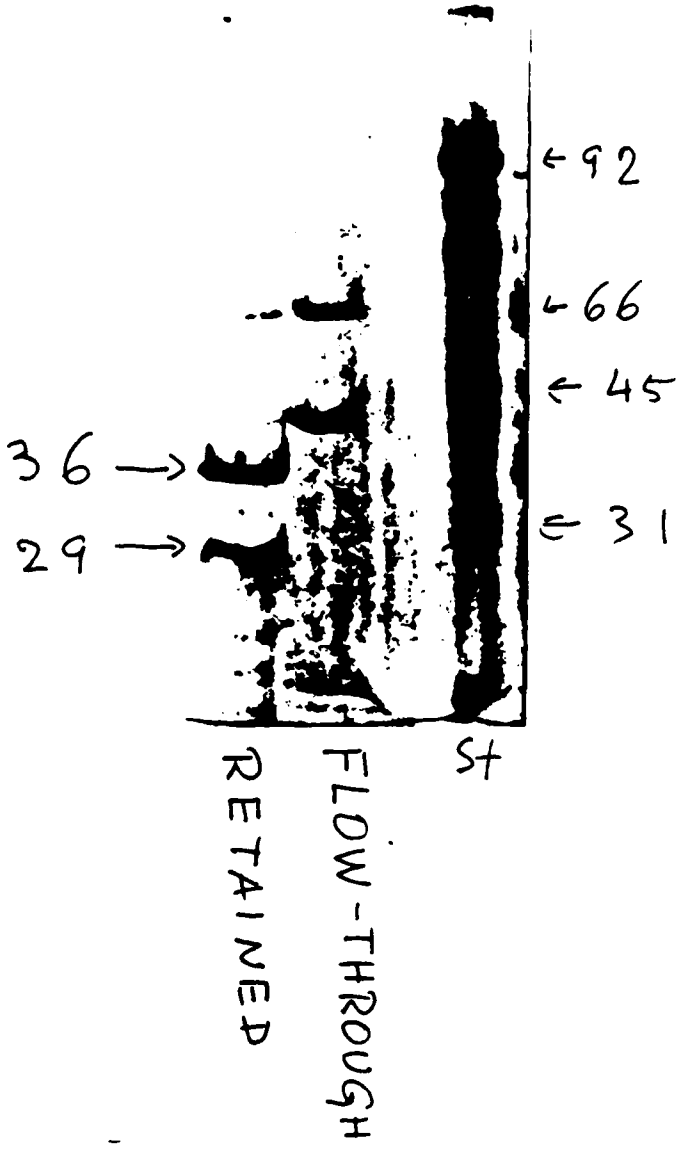
GURE 5: Digestion of the enzym with plasmin with plasmin in presence (+) and absence (-) of coenzymic DNA



Experimental conditions as in Fig. 3.

Fig 6

FIGU. 6: Separation of plasmin rele. ed peptides on DNA-cellulose. (DNA-binding assay).

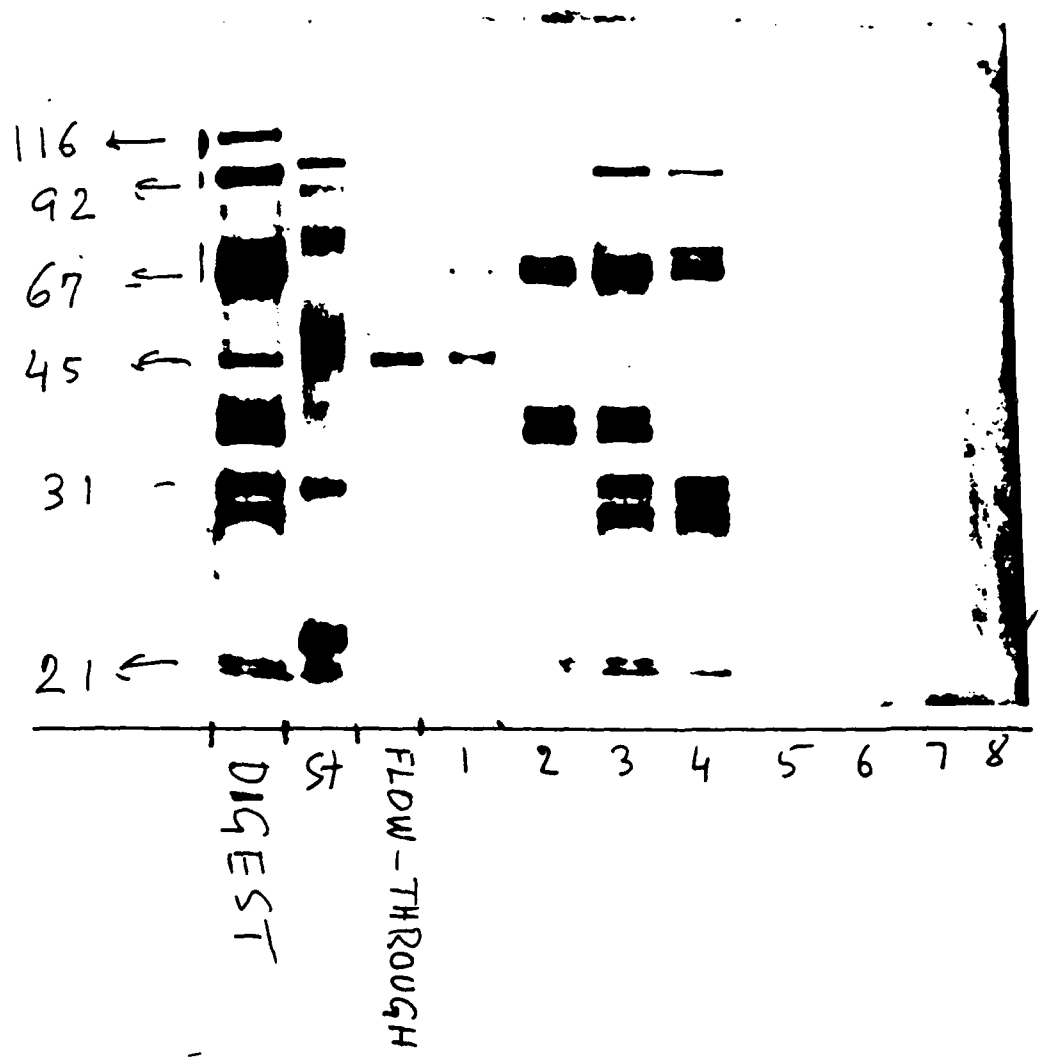


Digestion was done as given in Fig. 3, and reaction was stopped after 10 min. with 1 mM PMSF.

0.12 mg (43 µl) peptide mixture was applied to 1 ml DNA cellulose column, washed with buffer B containing 0.2 M NaCl; adsorbed peptide was eluted with the same buffer, containing 1 M NaCl. Flow-through and eluted peptides were applied to SDS-PAGE(10%) after concentration (Amicon) to 10-50 µl.

Fig 7.

FIGL 7: Chromatography of plasmin peptides on Red Sepharose.



Digestion was carried out as in Fig. 3 for 12 min. ($\frac{E}{protease}$ = 115); 0.8 mg (400 µl) digest was applied onto 1 ml Red-Sepharose (5.1 µmol/ml) in buffer B containing 15% ethylene glycol and 0.2 M NaCl. Elution with a salt gradient 0.2 - 2.0 M and 10 fractions were analyzed on SDS-PAGE (10%).

FIGURE 8. Isolation of Plasmin peptides by FPLC (MONO S)

Fig 8

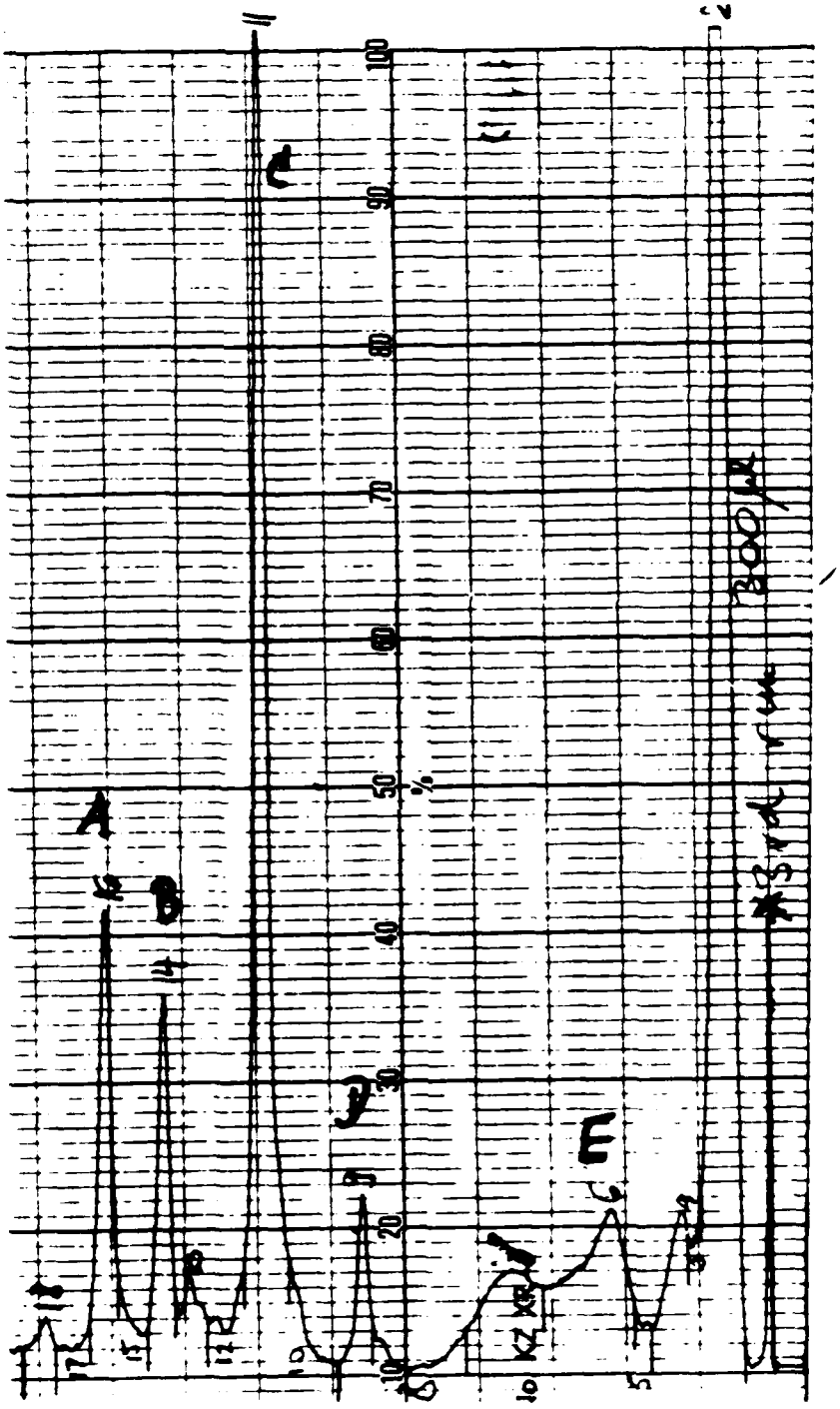


FIGURE 9. [illegible]-PAGE of MONO-S Fractions, sepa[illegible]ted by PAGE.

Fig 9.

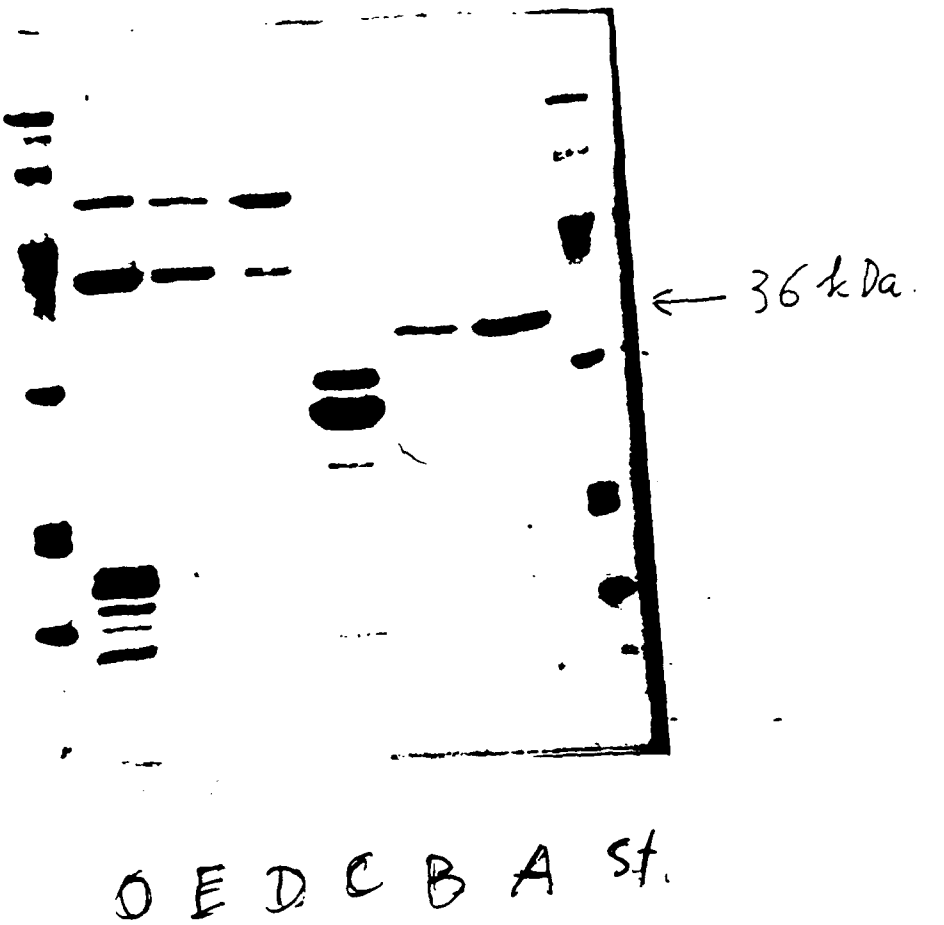


Fig 10

FIGURE . Antigenic properties of plas n peptides (immuno-transblot).

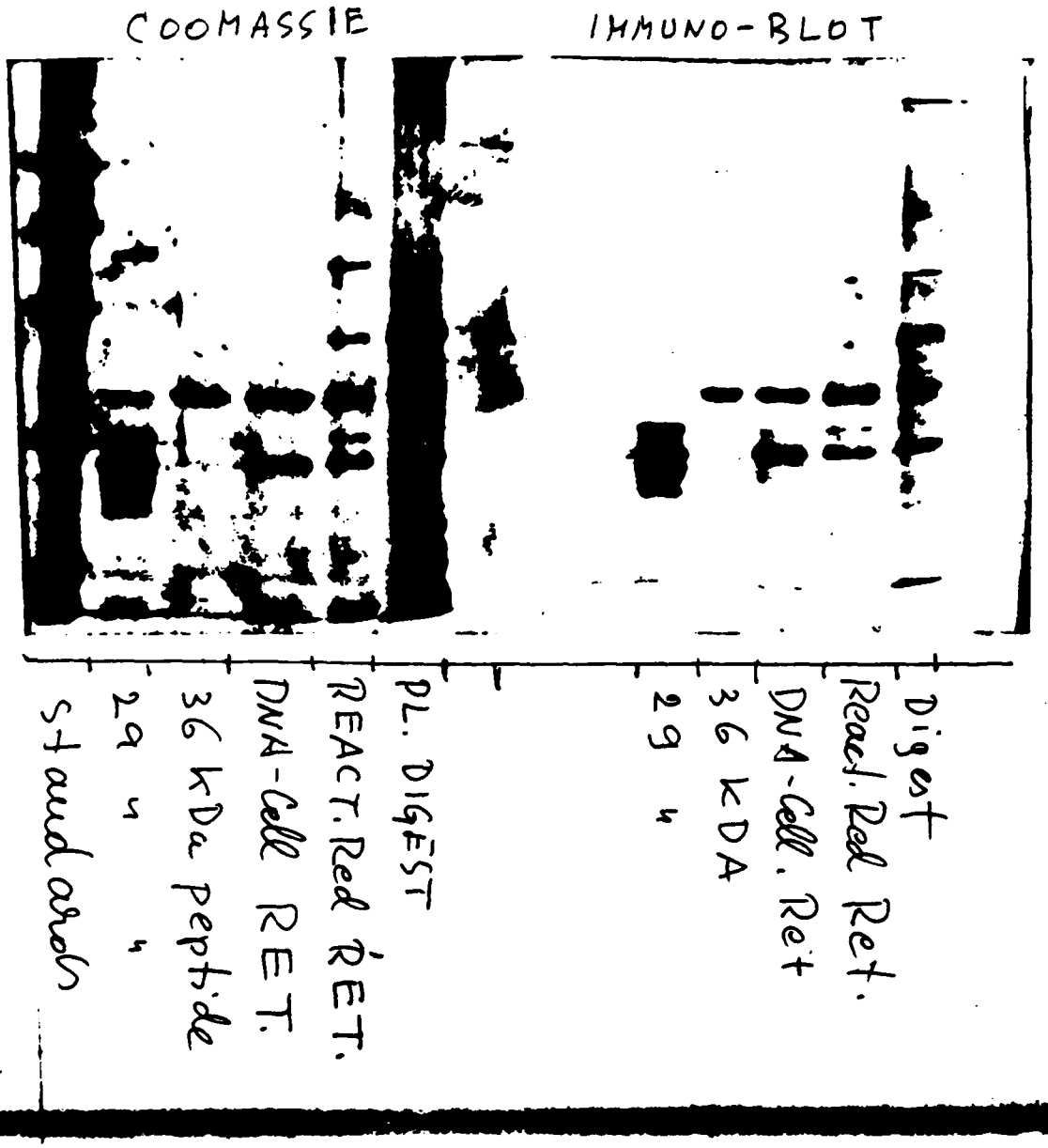


FIGURE 11

Partial amino-acid sequence of the "plasmin" peptide (Fig. 9, 36 kDa peptide), and computation of DNA sequences Probe 1 and 2.*

PROBE 1

AA NH_2	lys	ser	lys	lys	glu	lys	asp	lys	glu	ile	lys	leu	glu	lys	ala	leu								
mRNA 5'	AAG	AGC	AAG	AAG	GAG	AAG	GAC	AAG	GAG	AUC	AAG	CUG	GAG	AAG	GCU	CUG								
cDNA 3'	TTC	TCG	TTC	TTC	CTC	TTC	CTG	TTC	CTC	TAG	TTC	GAC	CTC	TTC	CGA	GAC								

PROBE 2

lys	ala	gln	asn	asp	leu	ile	trp	asn	val	lys	asp	glu	leu	lys	lys									
AAG	GCU	CAG	AAC	GAC	CUG	AUC	UGG	AAC	GUG	AAG	GAC	GAG	CUG	AAG	AAG									
TTC	CGA	GTC	TTG	CTG	GAC	TAG	ACC	TTG	CAC	TTC	CTG	CTC	GAC	TTC	TTC									

ala	~~lys~~	ser	thr	asn	asp	leu	lys	glu	(leu)	(leu)	ile	phe	asn	lys										
GCU	UGC	AGC	ACC	AAC	GAC	CUG	AAG	GAG	CUG	CUG	AUC	UUC	AAC	AAG										
CGA	ACG	TCG	TGG	TTG	CTG	GAC	TTC	CTC	GAC	GAC	TAG	AAG	TTG	TTC										

gln val pro -COOH
GAG GGG CCU 3'
CTC CAC GGA 5'

*These sequences were synthesized in September, 1986, and they are presently tested as gene-probes.

Amino acid sequences

The two 36 kd basic peptides - A and B on Fig. 8 - differ only by one amino acid at the N terminus. peptide B is one lysine shorter than A. The sequence of A peptide is as follows: KSKKE KDKEI KLEKA LKAQN DLIWN VKDEL KKA(C)S TNDLK E(LL)IF NKQUE P (amino acids in parentheses are not certain. (See also Figure 11).

The N-terminal amino acid sequence of the 42 kd peptide, which does not bind to either DNA cellulose or red-sepharose, is the following: VNPGT KSKLP KPVQN LIKMI FDVES MK(K)A. (See Fig. 11).

It is of interest that the 29 kd peptide - C of Fig. 8 - is not sequencable like the enzyme itself. The N-terminal of this peptide is not pyroglutamate, because pyroglutamate amino-peptidase treatment does not render the peptide sequencable. The blocking group is as yet unknown.

DISUCSSION

The main difficulty of presently available procedures for the isolation of poly(ADP-ribose) polymerase is a prevailing inconsistancy of purification steps. In particular the adsorption of the protein on DNA-cellulose proved to be variable and of low capacity. Another complication of the DNA-cellulose column is that it leaks DNA, and it is difficult to know whether or not "coenzymic" DNA tat is coisolated with the enzyme is not contaminated by DNA desorbed from DNA-cellulose. Zahradka and Ebisuzuki (1984) introduced two successive adsorption steps, first DNA-cellulose followed by Reactive Red 120-agarose, a procedure still dependent on the variable performance of DNA-cellulose. As we show here, the direct application of Reactive Red column as the first

adsorbent has distinct advantages and the column is reusable for a prolonged period. We find that if the Reactive Red contains lower substitution (2.7 μmol dye per ml packed gel) the enzyme can be adsorbed and eluted with aqueous buffers, but with a gel, containing higher concentration of the dye (5.1 μmoles red per ml packed gel) the enzyme was tightly adsorbed and was not elutable unless ethylene glycol was present. Ethylene glycol apparently prevents non-specific hydrophobic interactions between proteins and the dye, which can lead to irreversible denaturation of the enzyme (Seeling, Colman, 1977).

Another inconvenient step of purification is the final gel filtration which is time consuming, and unnecessarily dilutes the enzyme. Therefore, the purity of final product depends a great deal on conditions of gel filtration. Replacement of gel filtration by IPLC on MONO S cation exchanger greatly simplified and improved purification.

METHOD II

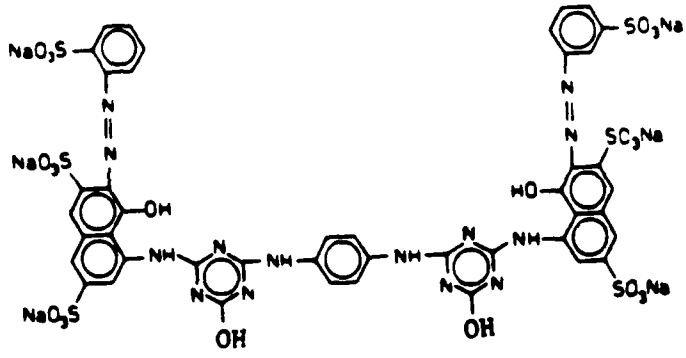
Affinity-precipitation of poly(ADP-ribose) polymerase.

Principle of the method:

While considerable amount of biochemical work was done with the enzyme protein , isolated by Method I, a second procedure was also developed based on a relatively new principle. Because of the success of "Red sephadex" column in procedure I, the affinity dye was directly tested as an enzyme precipitant, and unusually promissing results were obtained. In fact, Method II is far superior to any procedure used in this (or other) fields of enzymology and establishes the usefulness of a principle that was mostly a theory until now. The enzyme is effectively precipitated by the dye from a crude $(NH_4)_2SO_4$ cut, and purified to homogeneity in 3 steps (2-3 days) with a yield that exceeds

several fold at any previously employed procedure.

The chemical structure of the affinity dye is shown here:



Dihydroxy-Reactive Red 120

After precipitation of the dye-enzyme complex, the enzyme is extracted with KCl, when the K^+ salt of the dye forms an insoluble precipitate while the enzyme goes into solution. This is followed by hydroxy-apathite and FPLC chromatography, as shown in Table III.

Technical details of the procedure are as follows:

Buffers

Buffer I. 50 mM Tris-HCl, 10 mM EDTA, 1 mM NaN_3, 50 mM NaH SO_3, 10 mM mercaptoethanol, 300 mM NaCl pH 7.4.

Buffer II. 50 mM Tris-HCl, 1 mM EDTA, 1 mM NaN_3, 50 mM NaH SO_3, 10 mM mercaptoethanol, 10% glycerol, 15% ethylene glycol, pH 7.4.

Buffer III. 50 mM Tris, 1 mM EDTA, 1 mM NaN_3, 10 mM mercaptoethanol, 2 M KCl, 10% glycerol, pH 7.4.

The phenyl methylsulfonyl fluoride stock solution was 100 mM in

TABLE III

Isolation of the polymerase by affinity precipitation*

	Volume	Units/mg. prot.	Total Units	Total mg/protein	Protein Conc.
$(NH_4)_2SO_4$ fraction (40-80%)	300 ml	13	15,780	1200	4 mg/ml
Elution from dye precipitate	130	134	9,270	69	0.5
Eluate from hydroxylapatite	130	133	3,829	29	0.2
FPLC-fraction	0.28	986	1,972	2	7.0

*Preparation started with 250 g tissue (wet weight).

abs ethanol, and added to each buffer immediately before use to have 0.1 mM final concentration.

FPLC Buffers

Buffer A: 25 mM HEPES, 1 M NaCl.

Preparation of dihydroxy-Reactive Red 120 from the dichloro derivative.

It is of considerable importance to ascertain that the conversion of the Cl form of the dye to the OH form is quantitative. Two techniques are described to test for quantitative conversion.

Reactive 120 (Sigma #R0378) 2.2 g, were dissolved in 46 ml H_2O and 4 ml of 2 M NaOH was then added and the solution kept at 65°C until hydrolysis was complete. This was determined by (a) by taking aliquots for TLC analysis on silica gel developed with butane 2- propanol: ethyl acetate:water 20:35:10:35. After about 24 h there was no original die or monochloro-red to be detectable by TLC, only the slow-moving di-OH-red.

The dihydroxy-red was precipitated by adding four volumes of abs. EtOH, the precipitate centrifuged, washed successively with EtOH and ether, then dried. Yield: 1.6 g.

(b) An alternate procedure for the testing of the completion of hydrolysis of the chloro dye is based on elution of the OH dye from filter paper, whereas the chl-dye covenantly binds to cellulose. It is convenient to cut analytical filter paper into 1 cm^2 squares, which are placed into 0.5 ml 1 M $Na_2 CO_3$ which contains 0.2 ml aqueous solution of the dye to be tested. The entire system is kept at 65°C over night and the dye which was not adsorbed to the filter paper was removed, the filter paper washed with H_2O, and several changes of ethylene glycol.

If the dye sample contained the Cl-form, it was covalently bound to the filter paper, i.e., it could not be extracted.

Experimental procedures of enzyme isolation.

The extraction of frozen thymus tissue and preparation of the 40-70% (saturation) $(NH_4)_2SO_4$ cut is the same as in METHOD I.

The pellet containing the enzyme was dissolved in buffer II, and $(NH_4)_2SO_4$ concentration reduced by dilution with buffer II until conductivity (at 0°-4°C) reached 10-14 m Siemens. The volume at this state is 4-500 ml.

Affinity precipitation with dihydroxy Reactive Red from above obtained solution (containing 4 mg protein per ml) was done by adding $1/12^{th}$ volume of an aqueous solution of dihydroxy-reactive red (10 mg/ml) to achieve 0.06 mM final dye concentration) followed by stirring for 5 min. at 0-4°C. The red precipitate formed is sedimented at 7000 xg for 10 min. (at 4°C) and washed once with Buffer II containing 0.02 M NaCl. The red sediment, containing the enzyme-dye complex is suspended in 200 ml of Buffer III (containing 2 M KCl and 15% ethylene glycol). This treatment with Buffer III dissociates the enzyme from the dye - and precipitates the K^+ salt of the dye as an H_2O insoluble sediment the bulk of wich is removed by centrifugation (see above).

Adsorption of the enzyme (present in the extract with Buffer III, (see above) on hydroxylapatite and desorption.

10 g of hydroxylapatite (dye) is equilibrated in Buffer III (containing 15% ethylene glycol) for 10 min. (R.T.) and the slurry poured into a wide, short column (4 cm diameter, matrix of the hydroxylapatite = ~30 ml), then washed with 2 x 30 ml buffer III (+ 15% ethylene

FIGURE 12

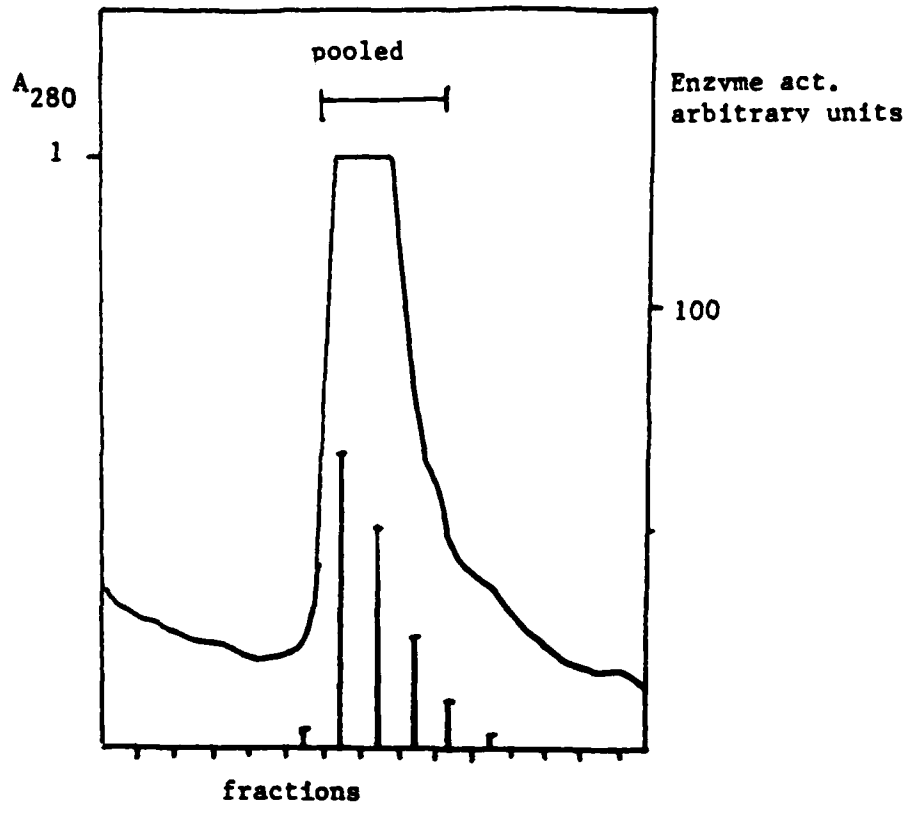


fig. Elution profile of poly(ADP-ribose) polymerase from hydroxyapatite

Hydroxyapatite column 3.4 x 4.4 cm, elution with buffer III containing 75 mM potassium phosphate, 6 ml fractions collected. Bars represent enzymatic activity, solid line is UV trace

FIGURE 13

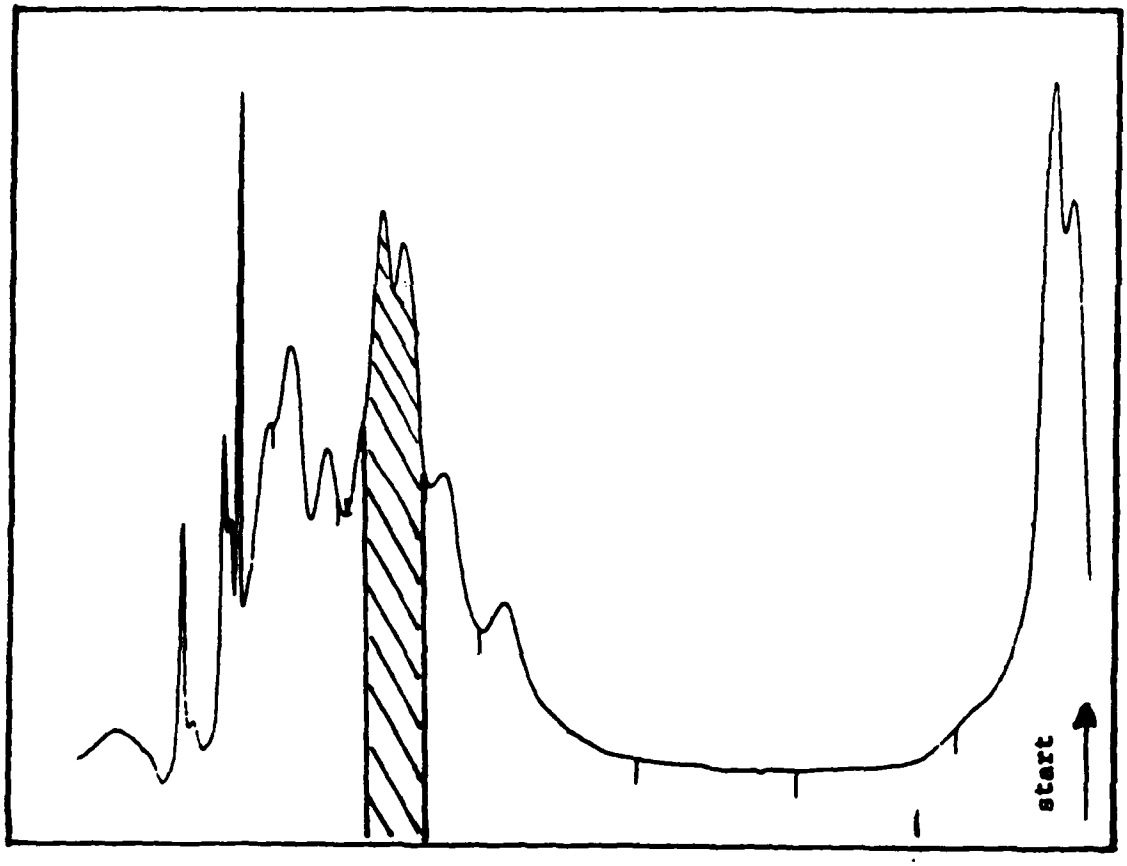


fig. Elution profile of poly(ADP-ribose) polymerase from FPLC column

Mono S 5/5 (Pharmacia) cationexchanger column; elution with 1.25% increase of buffer B per min; flowrate 0.8 ml per min. The shaded area collected as the enzyme

glycol). The enzyme is subsequently eluted with Buffer III. to which 75 mM K-phosphate (pH 7.4) has been added. The phosphate elutes the enzyme and some of the red dye, which is largely removed by a 0.9 cm x 10 cm cellulose column. The elution profile of the hydroxylapatite column is shown in Figure 12.

Final step: FPLC chromatography.

The enzyme containing fractions (faintly pink) are pooled and concentrated in an amino concentrator with PM 30 filter, simultaneously replacing Buffer III with Buffer A of the FPLC system. The concentrated enzyme solution was injected into a cation exchange FPLC system (MONO-S 5/5) and developed with Buffer B at a linear gradient of 1.25% per minute at a flow rate of 0.8 ml/min.

The elution of the enzyme is shown in Figure 13.

The resulting enzyme protein after concentration in a Centricon 30 is 90-95% homogeneous (3-10 mg protein/ml) which is sufficient for most enzymological experiments. If near 100% purity is desired the batch - elution of the enzyme from the hydroxylapatite column by 75 mM phosphate is replaced by a phosphate gradient, that eliminates contaminated proteins.

References

1. M. Bradford, Anal. Biochem. 72, 248 (1976).

2. W. N. Burnette, Anal. Biochem. 112, 195-202 (1981).

3. H.J. Burtscher, B. Auer, H. Klocker, M. Schweiger and M. Hirsch-Kauffmann, Anal. Biochem., 152, 286-290 (1986).

4. J. Holtlund, R. Jemtland, T. Kristensen, Eur. J. Biochem. 130, 309-314 (1983).

5. M. W. Hunkapiller, K. M. Hewick, W. J. Dreyer and L. E. Hood, Methods in Enzymol., Vol. 91, p. 399 (1983).

6. S. Ito, Y. Shizuta, O. Hayaishi, J. Biol. Chem. 254, 3647-3651 (1979).

7. I. Kameshita, M. Matsuda, M. Nishikimi, H. Ushiro, Y. Shizuta, J. Biol. Chem. 261(8), 2863-3868 (1986).

8. M.K. Laemmli, Nature 277, 680-681 (1970).

9. P.H. Pekala, and J. Moss, Curr. Top. Cell. Regul., Vol. 22, pp. 1-49 (1983).

10. G. F. Seeling, R.F. Colman, J. Biol. Chem. 252, 3671-3678 (1977).

11. K. Yoshihara, T. Hashida, Y. Tanaka, H. Ohgushi, H. Yoshihara and T. Kamiya, J. Biol. Chem. 253, 6459-6466 (1978).

12. P. Zahradka, K. Ebisuzaki, Eur. J. Biochem. 142, 503-509 (1984).

CONCLUSIONS

The first phase of experiments aimed at the isolation of the poly(ADP-ribose) polymerase gene consisted of:

a. Development of improved methods of enzyme isolation.

b. Isolation of a sequencable peptide that binds DNA, by plasmin proteolysis and sequencing of 51 amino-acids.

c. Identification and total synthesis of two DNA probes (see p.26), which are now being applied to identify the gene in DNA-libraries. Note: c. is a very recent accomplishment and therefore only net results are given (synthesis of DNA probes was completed on September 22, 1986.)

END

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